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Optimisation of culture of *Mycobacterium avium* subspecies *paratuberculosis* from blood samples

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ABSTRACT

Mycobacterium avium subspecies *paratuberculosis* (MAP) is the causative agent of Johne's disease or paratuberculosis, a chronic enteritis of ruminants, and has been suggested to play a role in Crohn's disease in humans. While disease expression is primarily in the gastrointestinal tract, isolation of MAP or MAP DNA at distant sites indicates that disseminated infections also occur. This is the first study to compare, optimise and critically evaluate different methods of concentrating MAP from spiked blood samples to enable its culture. Whole blood, erythrocytes, but not plasma, delayed the growth of MAP in BACTEC 12B medium. Culture of MAP from buffy coats (concentrated leukocytes) after lysis of erythrocytes and washing of the cells was an effective preparatory method. Several antibiotics were evaluated to reduce contamination of the slow growing MAP cultures with microbes which were derived from the skin during venipuncture, but were detrimental to the growth of MAP. However, decontamination of erythrocyte-lysed washed buffy coat samples in 0.75% hexadecylpyridinium chloride (HPC) for 72 h prior to inoculation of culture media did not inhibit the growth of MAP. The prepared samples can be stored at −80 °C prior to batch culture. MAP was isolated from the blood of 2 of 23 sheep 20 months after experimental inoculation. The optimised method has an analytical sensitivity of at least 10^1 MAP per ml of spiked whole blood and will enable trials to determine the incidence, duration and magnitude of mycobacteraemia in infected animals and humans.

1. Introduction

Mycobacterium avium subspecies *paratuberculosis* (MAP) is the causative agent of Johne's disease or paratuberculosis, which is widespread in both domestic and wild ruminants internationally. Infection results in a chronic enteritis with a long subclinical phase during which MAP may be shed in faeces. Clinical signs include emaciation, diarrhoea in some species, decreased milk production, decreased reproductive performance and increased mortality (Clarke, 1997). Paratuberculosis is an economically significant disease in cattle, sheep, goats and deer (Bush, 2005; Hutchinson, 1996; Losinger, 2005; Ott et al., 1999; Stehman, 1996). It may also present a risk to human health as MAP has been associated with Crohn's disease (Naser et al., 2004; Schwartz et al., 2000). However, there is considerable debate, and methods for sensitive detection of MAP will be essential to enable further investigation of this issue.

The detection of DNA specific to MAP in the blood of clinically and subclinically infected sheep and cattle (Barrington et al., 2003; Bhide et al., 2006; Buergelt et al., 2004; Buergelt and Williams, 2004; Gwozdz et al., 1997; Gwozdz et al., 2000; Juste et al., 2002; Juste et al., 2005) and the occurrence of disseminated infection (Antognoli et al., 2008; Hines et al., 1987) indicate that viable MAP may be present in the blood of some infected animals. However, few published data are available on the culture of MAP from the blood of infected animals or humans. MAP has been cultured from mononuclear cell-rich portions of blood, isolated using gradient centrifugation, from 1 of 7 clinically infected cattle (Koenig et al., 1993). In humans with Crohn's disease, MAP or MAP DNA has been detected from blood samples by culture (Chamberlin and Naser, 2008; Naser et al., 2004) and PCR (Juste et al., 2008; Juste et al., 2009). However, the presence of DNA does not indicate whether bacteria are viable. Thus, the incidence, intensity and duration of mycobacteraemia are not known in any animal species. If present, the mycobacteraemia may be continuous or intermittent. To enable reliable detection of MAP bacteraemia, a method for isolating and culturing MAP from blood has to be developed and evaluated. Detecting MAP in the blood may aid diagnosis of paratuberculosis and lead to a better understanding of the pathogenesis of the disease, especially disseminated infection, in all susceptible animal species.

Several methods of processing human and animal blood for culture have been described (David et al., 2004; Gill et al., 1985; Kiehn and Cammarata, 1986; Thornton et al., 1999; Zierdt, 1986; Zierdt et al., 1982) as have methods to process blood samples for detection of MAP DNA (Barrington et al., 2003; Buergelt et al., 2004; Gwozdz et al., 1997; Gwozdz et al., 2000; Juste et al., 2002; Juste et al., 2005; Ozbek et al., 2003). However, none of these methods have been critically evaluated, compared or optimised.

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Contamination is reported to be a problem in the culture of MAP from tissue and faecal samples (Dundee et al., 2001; Gumber and Whittington, 2007; Gwozdz, 2006; Nielsen et al., 2004; Reddacliff et al., 2003c; Whittington, 2009; Whittington et al., 1999). Contamination has also been a problem for the culture of blood samples from both cattle and humans, despite the apparent lack of gross contamination of the sample (Koenig et al., 1993; Vetter et al., 2001). This suggests that decontamination of blood samples prior to inoculation or during incubation in the media may be essential for isolation of MAP. However, this may reduce the sensitivity of the culture method (Grant et al., 2003; Gumber and Whittington, 2007; Reddacliff et al., 2003a).

The aims of this study were to develop a practical and effective method for processing blood samples to concentrate MAP prior to culture, and to evaluate the effects of antibiotics and decontamination procedures on the growth of MAP from blood samples. The highly sensitive BACTEC radiometric technique was employed; it is representative of other liquid culture systems. Isolates of the two predominant strains of MAP, S and C, were included in the study. Sheep blood was used in the experimental model, with spiking and incubation of the blood so that phagocytosis by leukocytes could occur, as this is likely in vivo.

2. Materials and methods

2.1. Animals and blood collection

All animal trials were approved by the University of Sydney Animal Ethics Committee. Blood was collected from unvaccinated sheep that were faecal culture and MAP antibody ELISA (Institut Porquier, Montpellier) negative. Sheep were originally sourced from properties which were considered unlikely to be infected with MAP. Sheep were housed under field conditions at the University of Sydney Farms (Begg et al., in press). Initially, blood was collected in citrate phosphate dextrose adenine anticoagulant (CPDA) (Baxter). In later experiments, blood was collected into 9 ml lithium heparin vacuum blood tubes (Vacutainer, Greiner Bio-one). The wool was clipped and the jugular groove cleaned with 70% v/v ethanol immediately prior to venipuncture to reduce the risk of contamination.

2.2. MAP strains and their culture

Telford 9.2 batch 2636-2, a clonal sheep (S) strain of MAP and CM00/416, a cattle (C) strain were used. For each experiment, a frozen (−80 °C) stock bacterial suspension previously enumerated using an end point titration method (Reddacliff et al., 2003b) was thawed and diluted to give the required concentrations of MAP. All samples were cultured using modified BACTEC 12B culture medium (Whittington et al., 1999). Cultures were incubated at 37 °C for 12 weeks. Analysis of growth was based on the number of days required to obtain a cumulative growth index of 1000 (dCGI1000) (Reddacliff et al., 2003b).

2.3. Phagocytosis of MAP by peripheral blood leukocytes

To allow for interactions such as phagocytosis between MAP and leukocytes, 9 ml of blood was spiked with MAP and incubated at 37 °C on a rotating mixer for 1 or 4 h. A non-spiked control was examined at time zero. After 0, 1 or 4 h, the WBC enriched fraction was obtained using the red cell lysis method described below and resuspended in 100 µl of phosphate buffered saline (PBS). Smears were created and stained using Romanowski and Ziehl Nielsen (ZN) stains and were examined under 1000× magnification to evaluate the extent of phagocytosis of MAP by leukocytes.

2.4. Preparation of spiked blood

100 µl of a bacterial suspension was added per 9 ml blood in a 50 ml tube (Falcon, BD). One negative control, to which sterile PBS was added, was included for each method. The blood samples were then incubated at 37 °C for 4 h on a rotary mixer at 2 rpm, subdivided into 9 ml aliquots and processed by the methods described below.

2.5. Pre-culture processing methods to concentrate MAP

All procedures were conducted using aseptic technique in a Biological safety cabinet, class 2. Unless otherwise described, the pellet produced by each method was resuspended in 100 µl of PBS and inoculated into BACTEC medium.

2.5.1. Isolator 10® tube lysis centrifugation (Oxoid)

An ISOLATOR 10® tube (Oxoid), which contains 0.7 ml of a sterile aqueous solution of 8 ml/l polypropylene glycol, 15.3 g/l sodium polyanetholsulphonate and 28 mg/ml purified saponin, was filled with 9 ml of blood then processed according to the manufacturer's instructions with the exception of centrifugation which was for 30 min at 3000 *g* with no brake. The resulting concentrate was mixed thoroughly by vortexing. Then either 0.2 ml was inoculated into BACTEC medium, or 0.2 ml, 1 ml or 1.5 ml was washed prior to inoculation to remove potential inhibitors to growth, by adding 1 ml of sterile PBS then centrifuging at 16,100 *g* for 10 min.

2.5.2. Saponin lysis centrifugation

Erythrocytes and leukocytes were lysed by adding 0.7 ml of 5% w/v saponin (Sigma) in sterile water to 9 ml of blood, inverting the tubes several times and incubating at room temperature for 10 min. After centrifuging these tubes at 3000 *g* for 30 min with slow brake, the upper 7–8 ml of supernatant was removed using a sterile pipette leaving 1–1.5 ml of concentrate which was mixed on a vortex mixer before transferring to a 1.5 ml tube (Eppendorf). This was centrifuged at 16,100 *g* for 10 min and the supernatant was discarded. The concentrate was washed with 1 ml PBS as described above and termed the lysis pellet.

2.5.3. Carboxypropylbetaine (CB-18) lysis centrifugation

40 ml of 1% v/v CB-18 100× stock (Integrated Research Technology, Baltimore), 5% v/v 20× Tris Citrate buffer pH 6.0 (Integrated Research Technology, Baltimore) and 0.25% w/v N-acetyl-L-cysteine (NALC) (Sigma) was added to 5 ml of blood and processed according to the manufacturer's directions. The sediment produced was transferred to a 1.5 ml tube, pelleted by centrifugation at 5000 *g* for 10 min, the supernatant was discarded and the pellet was resuspended in 100 µl of sterile water.

2.5.4. Crude buffy coat

9 ml of whole blood was centrifuged at 1455 *g* for 20 min with slow brake, and the leukocyte layer (buffy coat) was collected using a sterile disposable polypropylene bulb pipette and transferred to a 1.5 ml tube. Cells were pelleted at 200 *g* for 10 min, the supernatant was removed and the pelleted cells were resuspended in 100 µl of sterile PBS.

2.5.5. Ficoll gradient centrifugation

The buffy coat was collected as previously described, (de Silva et al., 2010) resuspended with 1 ml sterile PBS, transferred to a 1.5 ml tube and centrifuged at 200 *g* for 10 min. The supernatant was discarded and the cell pellet was resuspended in 100 µl of PBS.

2.5.6. Erythrocyte-lysed buffy coat

The crude buffy coat was collected as described above and combined with 5 ml erythrocyte lysis buffer (0.83% w/v NH_4Cl, 0.1% w/v $KHCO_3$,